Human VNO Receptor (R1)

5   **Field**

The present invention relates generally to the field of receptors, specifically

pheromone receptors, and more specifically to human vomeronasal organ receptors.

Background

Small, volatile and non-volatile organic molecules, commonly referred to as

10   pheromones, mediate species-specific chemical communication between terrestrial

animals. Pheromones are present in the secretions and excretions from various organs

and tissues, including the skin, and represent diverse families of chemical structures.

Pheromones play essential roles in sexual activity, reproductive biology, and other innate

animal behaviors (Luscher et al., (1959) Nature 18:55-56; Meredith (1983) *in*

15   Pheromones and Reproduction in Mammals (Vandenbergh, ed.) pp. 199-252, Academic

Press; Stern et al., (1998) Nature 392:177-179; Wysocki, (1979) Neurosci. Biobehav.

Rev. 3:301-341; Jacob et al., (2000) Hormones and Behavior 37:57-78; Grosser et al.,

(2000) Psychoneuroendocrinology 25:289-299). Some, but not all, terrestrial vertebrates

detect pheromones in the vomeronasal organ (the VNO), also known as Jacobson's organ,

20   a small dead-end tubular structure with an opening into the nasal cavity that is located

bilaterally at the base of the nasal septum (Moran et al., (1991) J. Steroid Biochem.

Molec. Biol. 39:545-552.).

The VNO was first identified in humans in 1703 but it was believed to be a

vestigial organ without function in the adult. In the 1990s, the presence of a VNO was

established, caudal to the nasal septal cartilage on both sides of the nasal septum, in more

than 1700 normal male and female human subjects (Berliner, (1996) J. Steroid Biochem.

Molec. Biol. 58:1-2; Gaafar et al., (1998) Acta Otolargyngol. 118:408-412; Smith et al.,

(1998) Micro. Res. Tech. 41:483-491) The VNO is physically separate and functionally

5   distinct from the olfactory epithelium that detects the volatile odorants. Odorants do not

bind to the VNO receptors.

The VNO is lined with neuroepithelial cells with a microvillar surface that is the

presumptive site of pheromone receptors. Immunohistochemical staining of adult human

VNO epithelium detects neuron-specific enolase and protein gene product (PGP) 9.5,

10   both neuronal and neuroendocrine markers, in some bipolar cells with morphological

similarities to olfactory receptor neurons (Takami et al., (1993) Neuroreport 4:375-378).

More recent studies show that the majority of the cells lining the lumen of the human

VNO stain with antibodies to synaptophysin or chromogranin which are also markers for

neuronal and neuroendocrine cells. These data provide clear evidence for the existence of

15   a neuroepithelium in the human VNO. However, Takami et al. (1993) do not detect

olfactory marker protein (OMP) in the human VNO even though it is expressed in the

VNO of other vertebrates including rodents. This may reflect an important and

interesting species difference between humans and other vertebrates.

In animals, signals from the olfactory epithelium travel via the olfactory bulb to

20   the olfactory cortex and then on to other regions of the brain. In contrast, signals from the

VNO are transmitted through the accessory olfactory bulb to the amygdala and

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hypothalamus (Broadwell et al., (1975) J. Comp. Neurol. 163:329-346; Kevetter et al.,

(1981) J. Comp. Neurol. 197:81-98). Surgical ablation of the VNO in male rodents alters

a variety of endocrine-mediated responses to female pheromones including androgen

surges, vocalization, territorial marking, and inter-male aggression. Ablation of the VNO

5   in female rodents delays or prevents activation of reproduction, abolishes the effects of

over-crowding on sexual maturation, and reduces maternal responses to intruders

(Wysocki et al., (1991) J. Steroid Biochem. Molec. Biol. 39:661-669). In humans, the

defect(s) that causes the inherited hypogonadal disorder, Kallmann Syndrome, is also

associated with defective development of the VNO-terminalis complex (Kallmann et al.,

10   (1943) Am. J. Ment. Defic. 48:203-236).

Application of only femtomole quantities of any of several proprietary, synthetic

vomeropherins directly to the VNO of human volunteers rapidly induces reproducible

negative voltage potentials that can be measured locally with a multifunctional miniprobe.

The electrophysiological response in the VNO is characteristic of a mass receptor

15   potential. The magnitude of the response is dose-dependent and is accompanied by

changes in autonomic nervous system function, brain wave activity, gonadotropin

secretion, and mood (Berliner et al., (1996) J Steroid Biochem, Molec. Biol. 58:259-265;

(1998a) J. Steroid Biochem. Molec. Biol. 65:237-242; Monti-Bloch et al., (1998b) Ann.

N.Y. Acad. Sci. 855:373-389; Monti-Bloch et al., (1994) Pyschoneuroendocrinology

20   19:673-686; Monti-Bloch et al., (1991) J. Steroid Biochem. Molec. Biol. 39:573-582;

Grosser et al., (2000) Psychoneuroendocrinology 25:289-299).

Recent fMRI studies detect dose-dependent activation of the anterior medial thalamus, the inferior frontal gyrus, and other regions of the human brain, in the absence of detectable odor, following administration of estra-1,3,5(10),16-tetraen-3yl acetate (PH15) to human volunteers. Although Sobel et al. ((1999) Brain 122:209-217) deliver

5   the compound non-specifically to the nasal cavity in these fMRI tests, Monti-Bloch et al. (1994) have demonstrated that this compound induces physiological responses *in vivo* only when applied specifically to the VNO but not when applied to either olfactory or respiratory epithelium of human subjects. Therefore, the fMRI data support the existence of a functional neurological connection between the VNO and the human brain which can

10   be activated by a vomeropherin.

Administration of naturally occurring compounds of known structure such as estra-1,3,5(10),16-tetraen-3-ol and androsta-4,16-dien-3-one to the human VNO induce bradycardia, bradypnea, increases in core body temperature, and other physiological responses. Stern et al. (1998) have demonstrated that odorless human pheromones,

15   obtained from the axillae of women at different stages of the menstrual cycle, exert opposing effects on ovulation when applied above the lips where they can volatilize into the nasal cavity of recipient females. Some vomeropherins act exclusively in human females or in males, and others exert opposite effects on autonomic reflexes such as body temperature. Taken together, these data provide substantial support for the existence of a

20   functional VNO in humans with the capacity to exert significant physiological effects *in vivo*.

The VNO system affords the unique opportunity to develop and market novel therapeutics to treat disease via previously unexploited targets and neurological pathways. This approach has substantial benefits for the patient over existing therapies including: (i) the ease of delivery to the VNO, (ii) the requirement for only picograms of drug, (iii) the

5   rapid response to drug, and (iv) the apparent absence of the side-effects and toxicity frequently associated with systemic (*e.g.*, oral) delivery of drug. Thus, targeting receptors in the human VNO for the treatment of disease is desirable.

The standard bioassay for screening candidate vomeropherins requires the participation of human volunteers because pheromones are species-specific. In this assay,

10   the compounds are delivered directly to the VNO of volunteers under IRB-approved protocols, thus necessitating prior toxicological study of each candidate vomeropherin in rodents. This expensive and time-consuming process limits the number of compounds that can be tested and hampers the detailed structure-activity relationship (SAR) analyses that are essential to successful drug discovery.

15   Viable neuroepithelial cells may be harvested directly from the human VNO for testing *in vitro*. The harvested VNO cells retain their characteristic neuroepithelial morphology in culture and respond electrophysiologically to the application of vomeropherins *in vitro*, thereby demonstrating the existence of functional receptors in cells from the target tissue. Although this method still requires the participation of human

20   volunteers, it increases the screening throughput and decreases the number of animals required for toxicological studies. However, only a limited number of non-dividing cells

with a ~2-week life-span are obtained from each volunteer, and thus we require an

entirely new approach to meet the demands of modern high throughput drug screening

and SAR.

Several groups have cloned receptor cDNAs that are expressed exclusively in the

5   VNO of rats and mice, but, to date, no one has cloned human VNO receptor cDNAs. The

sequence of the cloned rodent receptor cDNAs indicates that they belong to the

superfamily of G protein-coupled receptors containing seven transmembrane domains,

but they are unrelated to any of the G protein-coupled receptors expressed in the olfactory

epithelium (Dulac et al., (1995) Cell 83:495-206; Herrada et al., (1997) Cell 90:763-773;

10   Matsunami et al., (1997) Cell 90:775-784; Ryba et al., (1997) Neuron 19:371-379; Saito

et al., (1998) Brain Res. Molec. Brain Res. 60:215-227). Database comparisons identify

motifs common to Ca^{2+}-sensing and metabotropic glutamate receptors in some of the

clones. The apparent lack of homology to olfactory receptors is consistent with the

observation that many vomeropherins are inactive when applied specifically to human

15   olfactory epithelium *in vivo*.

Each cloned rodent receptor messenger RNA (mRNA) is detected by *in situ*

hybridization in only a small number of neuroepithelial cells that are dispersed throughout

the rodent VNO, and it is likely that each cell expresses only a single receptor gene.

(Dulac et al., 1995; Herrada et al., 1997; Matsunami et al., 1997; Ryba et al., 1997; Saito

20   et al., 1998). Some of the cloned rodent receptors exhibit sexually dimorphic expression,

i.e., they are expressed differently in males or females.

The rodent VNO receptors are assigned to separate multi-gene families by two criteria: (i) the length of the extracellular (N-terminal) protein domain, and (ii) the isoform of the signal-transducing G protein co-expressed in the same cell. Receptors in the "V1R" family have a relatively short extracellular N-terminal domain and are

5   expressed primarily in cells that express a $G\alpha_i$ isoform of G protein. Receptors in the "V2R" family have a long extracellular N-terminal domain and are expressed primarily in cells that express a $G\alpha_0$ isoform of G protein. Differences at the N-terminus between the V1R and V2R families may reflect differences in the structure of the ligand and/or in the location of the ligand-binding domain. (Matsunami et al., 1997; Ryba et al., 1997; Krieger

10   et al., (1999 J. Biol. Chem. 274:4656-4662). Neuroepithelial cells expressing these distinct G protein isoforms are spatially segregated in the VNO in separate apical and basal longitudinal zones, suggesting that there is true physiological significance to the differences between the V1R and V2R receptor families.

Krieger et al. (1999) have recently shown that G protein-coupled receptors

15   expressed in the rodent VNO are functionally linked to signal transduction pathways. Their results demonstrate that volatile and non-volatile pheromonal components of male rat urine selectively activate the major $G\alpha$ protein subtypes (G_i and G_0, respectively) expressed in the VNO of female rats. The data imply that V1R family receptors, which are co-expressed with G_i, respond to volatile compounds whereas V2R family receptors,

20   which are co-expressed with G_0, respond to non-volatile protein components of urine.

Dulac and Axel (1995) estimate that, in total, the rat V1R family contains

approximately 35 candidate pheromone receptors; Herrada and Dulac (1997) and Ryba

and Tirindelli (1997) estimate that the rat V2R family contains an additional 100

receptors. Of the various rodent tissues tested, only mRNA from the VNO gives a

5   positive signal on northern blots probed with the cloned (^{32}P-labeled) pheromone

receptor cDNAs. At this limit of sensitivity, these results suggest that the pheromone

receptors are expressed exclusively (primarily) in the VNO. At the present time, it is not

known if each VNO receptor recognizes a distinct pheromone or if several receptors

recognize the same compound.

10   At reduced stringency, the cloned rodent VNO receptor cDNAs cross-hybridize to

human genomic DNA. Dulac and Axel (1995) detect approximately 15 human genes that

cross-hybridize to rat V1R family probes, and Herrada and Dulac (1997) detect an

additional ten human homologues that cross-hybridize to rat V2R family probes. The two

sequenced human V1R genomic DNA clones have ~40-50% identity with the closest rat

15   homologue. However, both human genomic clones have a stop codon in the putative

coding region and may thus be pseudogenes (Dulac and Axel, 1995). Nevertheless,

cross-hybridization suggests the evolutionary conservation of G protein-coupled receptors

in the VNO and thereby provides a means to isolate human receptor clones.

The presence of these pseudogenes does not preclude the existence of functional

20   human VNO receptor genes, especially in view of our assays with cells harvested directly

from the VNO (Monti-Bloch (1997) Chemical Senses 22:752). The past difficulties in

isolating, characterizing and cloning a VNO receptor reinforce our assertion that an

appropriate way to isolate functional clones of the human VNO receptors is via cDNA

prepared directly from the target tissue. In fact, Cao et al. ((1998) Proc. Natl. Acad. Sci.

USA 95:11987-11992) have successfully isolated homologues from a goldfish cDNA

5   library using probes based on the rodent receptor sequences even though that species

lacks a defined VNO. The presence of pseudogenes in the family has not prevented the

successful cloning of olfactory or VNO receptors from a variety of species and they

should present no greater obstacle to the cloning of human VNO receptors.

Rodriguez et al. (Nature Genetics 26:18-19; 2000) using a human genomic library,

10   detected a single gene expressed in human olfactory mucosa that is alleged to be a

homologue of the rodent V1R sequences. They failed, however, to demonstrate the

expression of the receptor in the VNO.

Thus, isolation and characterization of the human VNO receptors is desirable for

the development of new drugs, high throughput assays and characterization of the

15   receptors and their signal transduction pathways.

Summary
The present invention is directed to a human pheromone receptor.

In one aspect of the invention there is a mRNA sequence encoding a human VNO

20   receptor or a portion thereof.

In a second aspect of the invention there is provided a human VNO receptor cDNA

sequence or a portion thereof. In one embodiment, there is a DNA sequence encoding a

functional receptor. In a second embodiment there is a DNA sequence encoding a mutant

allele.

In an another aspect there is a vector comprising the human VNO receptor cDNA

sequence encoding a functional pheromone receptor. The vector is capable of directing

5   expression of the encoded receptor in an appropriate cell or cell line.

In a further aspect there is provided transformed cells expressing a functional

human VNO receptor. In a preferred embodiment, the cells are oocytes such as human,

mouse, xenopus, etc. transformed or injected with the expression vector.

In another aspect of the invention there is provided a cell culture expressing a

10   functional pheromone receptor.

In yet another aspect there is provided a high throughput drug screening assay.

In a further aspect there are provided ligands that bind a human VNO receptor.

The ligands may be either agonists or antagonists, naturally-occurring or synthetic. The

ligand may find use in the treatment of depression, anxiety, phobias, blood pressure, pain,

15   pre-menstrual syndrome, endocrine disorders, sleep disorders, alertness, and sexual desire

In yet a further aspect of the invention there is provided antibodies to the human

VNO receptor.

In another further aspect of the invention there is provided antisense

oligonucleotide sequences capable of binding to and blocking the expression of the

20   human VNO receptor. In a preferred embodiment the oligonucleotide binds the mRNA

encoding the human VNO receptor. In another embodiment the oligonucleotide binds the

DNA encoding the human VNO receptor.

In another aspect, there is provided a transgenic animal expressing the human

pheromone receptor. In another aspect there is a second allele of the gene for the receptor

5   that contains both a termination codon which, if expressed, would encode a truncated

form of the receptor, and a silent mutation which, if expressed, would not change the

encoded amino acid. In another aspect, there is a genetic test that will determine if a

subject will benefit from activation of the receptor, inhibition of the receptor or gene

therapy with a vector expressing the receptor.

10   In another aspect, there is provided an oligonucleotide probe capable of

hybridizing to and detecting RNA or DNA sequences that encode the receptor, or under

reduced stringency hybridization conditions, detect related sequences. Such related

receptor sequences may share 30%, 50% or a higher level of homology. In a final aspect,

there is an oligonucleotide probe capable of distinguishing between the functional and

15   mutant alleles of the human VNO receptor.

Description of the Figures
     Figure 1 is the nucleotide sequence of hVNO-R1.

     Figure 2 is the long form amino acid sequence of hVNO-R1. This form of hNVO-

20   R1 is translated using the first in-frame start codon.

     Figure 3 is the short form amino acid sequence of hVNO-R1. This form of hNVO-

R1 is translated using the second in-frame start codon.

Figure 4 is the long form amino acid sequence of hVNO-R1 with the seven

theoretical transmembrane domains indicated.

Figure 5 is the nucleotide sequence of the mutant hVNO-R1 allele.

Detailed Description

5   The invention will now be described in detail by way of reference only using the

following definitions and examples. All patents and publications referred to herein are

expressly incorporated by reference.

The present invention provides a human pheromone receptor. The receptor was

cloned from human female VNO-specific mRNA and identified in a VNO-specific cDNA

10   library, which are unique resources for the identification and isolation of genes expressed

in the VNO, specifically genes for pheromone receptors, ion channels and prospective

reagents for high throughput assays. Although the human female VNO has been used and

is described in detail herein, the male VNO may be subjected to the same methods and

procedures to yield similar mRNA and a cDNA library. Thus, identification and

15   characterization of pheromone receptors, as well as the sexually dimorphic pheromone

response, may be investigated. Additionally, cells expressing the pheromone receptor *in*

vitro may be utilized to screen drug candidates to identify agonists and/or antagonists.

Drugs so identified find a therapeutic use in psychiatric disorders and neuroendocrine

disorders and diseases. Those diseases and disorders include, but are not limited to,

20   premenstrual syndrome (PMS), premenstrual dysphoric disorder (PMDD), depression,

anxiety, alertness, sleep disorders, weight and appetite control, fertility, contraception, hormone regulation and the like.

Definitions

As used herein, the following terms or abbreviations, whether used in the singular

5   or plural, will have the meanings indicated:

"Antibody" means an immunoglobulin that specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of another molecule such as a human pheromone receptor. The antibody can be monoclonal or polyclonal and can be prepared by techniques that are well known in the art such as

10   immunization of a host and collection of sera (polyclonal) or by preparing continuous hybridoma cell lines and collecting the secreted protein (monoclonal), or by cloning and expressing nucleotide sequences or mutagenized versions thereof coding at least for the amino acid sequences required for specific binding of natural antibodies. Antibodies may include a complete immunoglobulin or fragment thereof, which immunoglobulins include

15   the various classes and isotypes, such as IgA, IgD, IgE, IgGI, IgG2a, IgG2b and IgG3, IgM, etc. Fragments thereof may include Fab, Fv and F(ab'), Fab', and the like. In addition, aggregates, polymers, and conjugates of immunoglobulins or their fragments can be used where appropriate so long as binding affinity for a particular molecule is maintained.

20   An "expression construct" or "expression vector" is a vector which is constructed so that the particular coding sequence is located in the vector with the appropriate control

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sequences including regulatory sequences, the positioning and orientation of the coding

sequence with respect to the control sequences being such that the coding sequence is

transcribed under the "control" of the control or regulatory sequences. Modification of

the sequences encoding the particular protein of interest may be desirable to achieve this

5   end. For example, in some cases it may be necessary to modify the sequence so that it

may be attached to the control sequences with the appropriate orientation; or to maintain

the reading frame or to target the encoded protein to the correct subcellular location such

as the plasma membrane. The control sequences and other regulatory sequences may be

ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding

10   sequence can be cloned directly into an expression vector which already contains the

control sequences and an appropriate restriction site which is in reading frame with and

under regulatory control of the control sequences.

A "ligand" is a molecule that binds to another molecule, used especially to refer to

a small molecule that binds specifically to a larger molecule, e.g., an antigen binding to

15   an antibody, a hormone or neurotransmitter binding to a receptor, or a substrate or

allosteric effector binding to an enzyme. Ligand may be used interchangably with agent.

A "pheromone" is a biochemical produced by an animal or individual which elicits

a specific physiological or behavioral response in another member of the same species.

In addition to physiological responses, pheromones can be identified by their species

20   specific binding to receptors in the vomeronasal organ (VNO). Thus, human pheromones

bind to human receptors. This can be demonstrated by measuring the change in the

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summated potential of neuroepithelial tissue in the presence of the pheromone. Human

pheromones induce a change of at least about -5 millivolts in human neuroepithelial

tissue of the appropriate sex (The binding of pheromones is generally sexually

dimorphic.). Naturally occurring human pheromones induce sexually dimorphic changes

5   in receptor binding potential *in vivo* in the human VNO. Naturally occurring human

pheromones can be extracted and purified from human skin and they can also be

synthesized. "Human pheromones" are pheromones that are naturally occurring in

humans and effective as a specifically binding ligand in human VNO tissue, regardless of

how the pheromone was obtained. Thus, both a synthesized and purified molecule may

10   be considered a human pheromone. Commonly, pheromones affect development,

reproduction, mood, and related behaviors.

As used herein, an oligonucleotide probe is a DNA or RNA fragment that includes

a sufficient number of nucleotides to hybridize specifically to DNA or RNA that includes

identical or closely related sequences of nucleotides. A probe may contain any number of

15   nucleotides, from as few as 7 and as many as is desired. The conditions and protocols for

such hybridization reactions are well known to those of skill in the art as are the effects of

probe size, temperature, degree of mismatch, salt concentration and other parameters on

the hybridization reaction. For example, the lower the temperature and higher the salt

concentration at which the hybridization reaction is carried out, the greater the degree of

20   mismatch that may be present in the hybrid molecules.

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To be used as a hybridzation probe, the nucleic acid is generally rendered

detectable by labeling it with a detectable moiety or label, such as ^{35}S, ^{32}P, ^{3}H and ^{14}C,

or by other means, including chemical labeling, such as by nick-translation in the

presence of deoxyuridylate biotinylated at the 5'-position of the uracil moiety. The

5   resulting probe has incorporated the biotinylated uridylate in place of thymidylate and can

be detected based on the binding of streptavidin to the biotin moiety. Any other label

may be used as long as it renders the probes sufficiently detectable.

For the purposes herein, the following stringency conditions are defined:

1) high stringency: 0.1 x SSPE (1X SSPE = 150mM NaCl, 10 mM

10   NaH_2PO_4, pH 7.4, 1 mM EDTA), 0.1% SDS, 65°C

2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C

3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C

or any other combination of salt and temperature and other reagents that result in

selection of the same degree of mismatch or matching.

15   "Sexually dimorphic" refers to a difference in the effect of, or response to, a

compound or composition between males and females of the same species.

"Vomeropherin" as used herein is a more general term which includes pheromones

and describes a substance from any source which functions as a chemosensory

messenger, binds to a specific vomeronasal neuroepithelial receptor, and induces a

20   physiological or behavioral effect. The physiologic effect of a "vomeropherin" is

mediated through the vomeronasal organ. Vomeropherins may be naturally occurring

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compounds, synthetic modifications of natural compounds or totally synthetic

compounds.

The terms "vector" or "plasmid" as used herein mean a polynucleotide comprised

of single strand, double strand, circular, or supercoiled DNA or RNA capable of

5   replicating in a host cell and into which a coding sequence can be inserted. A typical

. vector may be comprised of the following elements operatively linked at appropriate

distances for allowing functional gene expression: replication origin, promoter, enhancer,

5' mRNA leader sequence, ribosomal binding site, nucleic acid cassette, termination and

polyadenylation sites, and selectable marker sequences. One or more of these elements

10   may be omitted in specific applications. .The nucleic acid cassette can include a

restriction site for insertion of the nucleic acid sequence to be expressed. In a functional

vector the nucleic acid cassette contains the nucleic acid sequence to be expressed

including translation initiation and termination sites. An intron optionally may be

included in the construct, preferably $\geq$ 100 bp 5' to the coding sequence. Vector and

15   plasmid are used interchangeably herein.

Cultured VNO cells are of limited value as a screening tool due to the need to

continually isolate new cells. Thus, it would be advantageous to clone and express

pheromone receptors in a cell line.

Normal human female and male VNO specimens were obtained by a team of

20   surgeons from donors, with informed consent, undergoing a surgical procedure necessary

to treat an injury, disease or other condition of the nasal septum. RNA was isolated from

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a pool of female VNOs or from individual female and male VNO specimens. Human

VNO RNA is essential because (i) the receptors are species-specific, (ii) the receptors

may be expressed exclusively in the VNO, and (iii) human genomic DNA contains

receptor pseudogenes and introns.

5   The pooled female VNO RNA was used to prepare a cDNA library. In brief, RNA

was extracted from pooled VNO specimens and reverse transcribed with SUPERSCRIPT II

reverse transcriptase (Life Technologies) to make first-strand cDNA using a Not I-

oligo(dT)$_{12-18}$ primer. *E. coli* DNA polymerase and RNase H were used for second-

strand synthesis. Sal I adapters were ligated to the ends and the double-stranded cDNA

10   was digested with Sal I and Not I. The cDNA was directionally ligated into pCMV-

Sport7.neo (Life Technologies) and transformed into *E. coli*.

        Certain vomeropherins elicit sexually dimorphic responses and some of the

receptors are expressed dimorphically. In consideration of these observations, we

constructed our VNO cDNA library with tissue obtained exclusively from human

15   females. Although others have successfully prepared cDNA libraries from individual

rodent VNO neuroepithelial cells, we used whole VNO tissue pooled from a number of

donors in order to maximize the number, size, and diversity of receptor clones in our

library. The library provides an excellent source to search for novel genes, gene

fragments, or other nucleotide sequences encoding proteins that are implicated in

20   detection of pheromones or other vomeropherins in the human VNO. Plasmid vectors are

currently available that can accommodate the directional cloning of cDNA such that T7

and SP6 RNA polymerase promoter sequences can be used to generate sense and

antisense transcripts for subtractive hybridization and riboprobe synthesis.

The GenBank human high throughput genome sequence (htgs) database was

searched for potential open reading frames capable of encoding a protein with homology

5   to one or more of the putative rodent pheromone receptors. Expression of the inventive

receptor nucleotide sequence in the human VNO was determined by a combination of

reverse transcription and polymerase chain reaction (RT-PCR) using RNA from

individual female and male VNO specimens and using cloned cDNA from a human

female VNO cDNA library.

10   The VNO RNA was reverse transcribed into first-strand complementary DNA

(cDNA) using AMV reverse transcriptase and a mixture of random hexamer primers.

The first-strand cDNA was used as the template in PCR reactions that were primed with

oligonucleotide pairs whose sequence was based on the targets identified in GenBank.

Positive results (i.e., synthesis and detection of an amplicon of the predicted size) were

15   obtained with both female and male VNO cDNA using the primer pair designed to

amplify a portion of the sequence designated hVNO-R1. PCR analyses with this primer

pair demonstrated that the target sequence was also represented in the female VNO

cDNA library. The entire predicted hVNO-R1 sequence was subsequently amplified

using the VNO first-strand cDNA as template by PCR with primers flanking the entire

20   open reading frame.

Screening techniques other than PCR, RT-PCR or hybridization are well known to

those of skill in the art and the selection of the techniques does not limit the present

invention. The procedures for isolating and identifying gene fragments are well known to

those of skill in the art; see, e.g. T. Maniatis et al, Molecular Cloning (A Laboratory

5   Manual), Cold Spring Harbor Laboratory Press.

The amplified DNA containing the complete open reading frame was cloned and

sent to Sequetech (Mountain View, CA) for sequencing on a standard fee-for-service

basis. The sequence of the receptor clone hVNO-R1 (SEQ ID No. 1) differs from the

corresponding GenBank human genomic DNA entry, and from a second hVNO-R1 clone

10   (designated hVNO-R1 Gen; SEQ ID No. 2), by only a single nucleotide difference

$(G \rightarrow A)$ at position 778. Nevertheless, both sequences encode the same protein due to the

known degeneracy of the genetic code. The nucleotide sequence (SEQ ID No. 1) has a

start codon beginning at position 17 which predicts a protein with the amino acid

sequence shown in Figure 2 (SEQ ID No. 3). Alignment of the amino acid sequence with

15   putative rodent pheromone receptors indicates that the closest rodent homologue may be

the rat VN6 receptor. The two protein sequences share 46% homology when

conservative amino acid changes are permitted. A second in-frame start codon at position

44 would give rise to a shorter version of the receptor (SEQ ID No. 4).

Alignment of hVNO-R1 (SEQ ID. No. 1) with the human V1RL1 sequence

20   (Rodriguez et al; 2000) shows that the two proteins share 37% homology when

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conservative amino acid changes are permitted. Thus, hVNO-R1 is a novel receptor with

some homology to other putative rodent and human pheromone receptors.

A second allele of the VNO receptor sequence (denoted as PP166; FIGURE 5;

SEQ ID No. 5) is also expressed as an RNA in the VNO of some subjects but differs from

5   hVNO-R1 (SEQ ID No. 1). A point mutation (C→T) at position 136 of SEQ ID No. 5

creates a termination codon, which, if translated into a protein, will yield a truncated form

of the receptor (SEQ ID No.6). The silent base difference (G→A) is also present at

position 762 of SEQ ID No. 5 but, due to the degeneracy of the genetic code, will not

alter the encoded amino acid if the sequence were expressed as a protein.

10   The existence of two alleles of the receptor sequence enables a genetic test to

determine whether a human subject will have a physiological response to administration

of either an agonist or antagonist ligand. Subjects who express only the alternative form

of the mRNA may not be expected to respond to administration of the ligand. Such

subjects may not experience a therapeutic benefit from the compound and could be

15   excluded from such trials and treatments and/or become candidates for gene therapy using

a vector that expresses a functional form of the receptor.

Once identified and sequenced, the nucleotide fragments of the genes of the

invention may be readily synthesized by conventional means such as solid phase oligo-

DNA synthesis (Letsinger et al., (1965) Oligonucleotide synthesis on a polymer support.

20   J. Am. Chem. Soc. 87:3526-3227). Alternatively, the DNA may be produced by

recombinant methods, then sequenced. Cloning procedures are conventional and are

described by T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring

Harbor Laboratory (1982). Further, hybridization or PCR methods can be performed

using known probes in order to determine whether or not a selected gene is expressed in a

gender specific manner may be performed.

5   Cloned probes which can be made starting from any DNA fragment according to

this invention, and thus to recombinant DNAs containing such fragments, are also

contemplated herein. Using the cloned DNA fragments as a molecular hybridization

probe - either by labeling with radionucleotides or with fluorescent reagents – differences

in the expression of the two alleles may be detected.

10   Oligonucleotide probes based on the VNO receptor gene sequence (SEQ ID No. 1)

capable of hybridizing to and detecting RNA or DNA sequences are also encompassed by

the present invention. Under stringent conditions the probes detect RNA or DNA

sequences encoding the receptor, or under reduced stringency hybridization conditions,

the oligonucleotide probes detect related sequences. Such related receptor sequences may

15   share a level of homology of preferably about 30%, more preferably about 50% or most

preferably about 75%.

Probes according to the invention can also be used for rapid screening of nucleic

acids derived from the tissue of patients, to see if the receptor DNA or RNA is present in

the patient's VNO and other tissues. An oligonucleotide probe capable of distinguishing

20   between the functional and mutant alleles of the human VNO receptor may also be used

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as a probe. These oligonucleotide sequences comprise the region surrounding the point

mutation at position 136 of SEQ ID No. 5 described herein.

A method which can be used for such screening comprising the following steps:

extraction of DNA from tissue, restriction enzyme cleavage of said DNA, electrophoresis

5   of the fragments and detection of single DNA base mutations with mismatch repair

enzymes (Lu et al., Genomics 14:249-55 (1992)) or screening for mutations by enzyme

mismatch cleavage with T4 endonuclease VII (Youil et al., Proc Natl Acad Sci USA

92:87-91 (1995)) and the like. Hybridization *in situ* can also be used.

The DNAs or DNA fragments according to the invention can be used also for

10   achieving the expression of human VNO pheromone receptors for drug discovery

purposes and the like.

Plasmids are DNA molecules that can be introduced or transfected into cells by

techniques well known in the art and which may be replicated either autonomously or

after integration into the genome of the host cell. The primary expression construct used

15   in the present invention comprises (i) a marker gene and (ii) control sequences operably

linked to the marker gene and sufficient for effecting expression of the marker gene in

competent cells, wherein the control sequences comprise at least one control sequence

from a gene which is substantially expressed only in the competent cells. Preferably the

cells are germline-competent, i.e., a cell which can contribute to germline tissue when

20   transgenic animals are desired. Typically, the control sequences sufficient for effecting

expression of the marker gene in competent cells may comprise multiple control

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sequences, such as a combination of a promoter and an enhancer. Alternatively, in other

embodiments, a single control sequence, such as a promoter, may be sufficient for

effecting expression of the marker gene in competent cells. The control sequence which

is from a gene that is substantially expressed only in cells which are competent may

5   optionally comprise an enhancer, a promoter, or both. Alternatively, the control

sequences of the expression construct may comprise a negative regulatory element which

is active only when a cell is differentiated. As an additional alternative, the coding

sequence may encode an antisense RNA molecule and could inhibit the production of

receptors that are involved in many disorders. The means of their action may be by

10   encoding or directly being antisense, thereby inhibiting the production of hVNO-R1.

Cell lines that stably or transiently express a VNO gene may be engineered. The

inventive VNO receptor gene sequence may be inserted into an expression plasmid

comprising a selection marker and suitable regulatory elements, and transfected into a

competent host cell. Following the introduction of the plasmid by methods known in the

15   art (for example, calcium phosphate precipitation, electroporation and the like),

engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are

switched to a selective media. The selectable marker in the novel plasmid confers

resistance to the selection and allows cells to grow to form foci which in turn can be

cloned and expanded into cell lines. This method may advantageously be used to

20   engineer cell lines that express the desired VNO gene product on the cell surface, and are

particularly useful in screening candidate drugs. For example, these cell lines are used to

develop automated high throughput screening assays for novel compounds, agents or

ligands with therapeutic utility in the treatment of psychiatric and endocrine disorders and

diseases such as, but not limited to: premenstrual syndrome (PMS), anxiety and phobias,

depression, alertness, sleep disorders, appetite control, blood pressure, pain, fertility, and

5   hypothalamic-pituitary disorders and the like.

The transformed cells may provide the basis for the development of transgenic

animals. Transformed germline-competent cells are introduced into an early stage

embryo, e.g. a normal blastocyst. Transgenic animals are preferably a mammal, more

preferably a rodent, and most preferably a mouse.

10   This method entails first introducing into embryonic cells an expression construct

which comprises a marker gene operably linked to control sequences sufficient for

effecting expression of the marker gene in germline-competent cells, wherein the control

sequences comprise at least one control sequence from a gene which is substantially

expressed only in cells which are germline-competent. Next, embryonic cells which

15   express the marker gene sequence are identified. These cells (typically about one

hundred or more) are then transferred to a recipient embryo, where the genome of the

recipient embryo differs from that of the transferred cell(s). In a preferred embodiment,

at least some of the cells which are transferred to the recipient embryo contribute to its

development. In a final step, the recipient embryo is allowed to develop at least until the

20   full gestational age is reached.

In another aspect of the invention there is provided antibodies for the receptor.

Once the receptor has been expressed, isolated and characterized it would be

advantageous to generate antibodies against the receptor. Antibodies to the receptor

would be useful in the treatment of diseases and disorders associated with the

5　overexpression of the receptor and to assess normal physiological function of the

receptor.

Antiserum containing antibodies (polyclonal) is obtained by well-established

techniques involving immunization of an animal, such as a rabbit, guinea pig, or goat,

with an appropriate immunogen and obtaining antisera from the blood of the immunized

10　animal after an appropriate waiting period.

Antibodies can also be obtained by somatic cell hybridization techniques, such

antibodies being commonly referred to as monoclonal antibodies. Monoclonal antibodies

may be produced according to the standard techniques of Köhler and Milstein, (Nature

265:495-497, 1975). Reviews of monoclonal antibody techniques are found in state-of-

15　the-art reviews are provided by Birch et al., (1995) Monoclonal Antibodies: principles

and application, Wiley-Liss, N.Y.; Davis (1995) Monoclonal antibody protocols, Humana

Press, Totowa, N.J. Samples of an appropriate immunogen preparation are injected into

an animal such as a mouse and, after a sufficient time, the animal is sacrificed and spleen

cells obtained. Alternatively, the spleen cells of a non-immunized animal can be

20　sensitized to the immunogen *in vitro*. The spleen cell chromosomes encoding the base

sequences for the desired immunoglobins can be expressed by fusing the spleen cells,

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generally in the presence of a non-ionic detergent, for example, polyethylene glycol, with

a myeloma cell line. The resulting cells, which include fused hybridomas, are allowed to

grow in a selective medium, such as HAT-medium, and the surviving immortalized cells

are grown in such medium using limiting dilution conditions. The cells are grown in a

5   suitable container, e.g., microtiter wells, and the supernatant is screened for monoclonal

antibodies having the desired specificity.

Various techniques exist for enhancing yields of monoclonal antibodies, such as

injection of the hybridoma cells into the peritoneal cavity of a mammalian host, which

accepts the cells, and harvesting the ascites fluid. Where an insufficient amount of the

10   monoclonal antibody collects in the ascites fluid, the antibody is harvested from the blood

of the host. Alternatively, the cell producing the desired antibody can be grown in a

hollow fiber cell culture device or a spinner flask device, both of which are well known in

the art. Various conventional ways exist for isolation and purification of the monoclonal

antibodies from other proteins and other contaminants (see Köhler and Milstein, *supra*).

15   In another approach for the preparation of antibodies the sequence coding for

antibody binding sites can be excised from the chromosome DNA and inserted into a

cloning vector which can be expressed in bacteria to produce recombinant proteins having

the corresponding antibody binding sites.

In general, antibodies can be purified by known techniques such as

20   chromatography, e.g., Protein A chromatography, Protein G chromatography, DEAE

chromatography, ABx chromatography, and the like, filtration, and so forth. Antibodies

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may be used as diagnostic tools such as to determine if an individual expresses the

receptor encoded by SEQ ID No. 3, 4 or 6. This information will be useful to determine

if a compound or drug which binds to the encoded receptor will have a physiological

effect and/or therapeutic utility in a subject. Other uses include applying the antibodies as

5   a therapeutic to block binding of a compound to the receptor on the cell surface or to

prevent the receptor from interacting with and/or transducing a signal to its binding

partners.

Also contemplated by the present invention are antisense oligonucleotides capable

of binding to complementary sequences and blocking the expression of the human VNO

10   receptor gene. The antisense oligonucleotides may be either RNA or DNA. The length

of antisense oligonucleotides depends, in part, on the target sequence, the desired

specificity and the base composition. Effective antisense oligonucleotides are preferably

at least 10, more preferably 15 or most preferably 20-30 bases long. Certain modified

oligonucleotides of only 7 bases can be effective antisense oligonucleotides (see Wagner

15   et al. (1996) Nature Biotechnology 14:840-844). Antisense oligonucleotides can contain

the "normal" bases with standard 5'-3' phosphodiester linkages or may contain modified

(e.g., methylated) bases, modified sugars (e.g., methylated), non-ribose sugars, or various

alternative linkages (e.g., phosphothioate, carbamate, peptide, alkylphosphonate,

phosphorimadate, acetamidate, etc.), and/or mixtures of normal/modified bases and

20   standard/alternative linkages. In another approach, an expression vector is contemplated

that, when introduced into cells by techniques well known in the art, directs the synthesis

of an antisense RNA that hybridized to complementary sequences in the cells.

The antisense oligonucleotides and antisense expression vectors may be used to

modulate expression of the receptor in a cell. Reduced receptor expression may be

5   desirable in various conditions and diseases including, but not limited to, fertility,

modulation of hormone levels or when the receptor is over-expressed.

Examples
The following preparations and examples are given to enable those skilled in the

10   art to more clearly understand and to practice the present invention. They should not be

considered as limiting the scope of the invention, but merely as being illustrative and

representative thereof.

15   Example 1
                                  Tissue Collection
Human VNO tissue specimens were collected for this purpose by a team of

surgeons. The human VNO is located bilaterally in the nostrils, and has been associated,

inter alia, with pheromone reception. The VNO is a small nasal organ with a central

20   lumen and a pit opening to the nasal cavity. The VNO is a bilateral structure located

supra palatial. The pit is approximately 1 to 1.5 mm in diameter and the lumen is

approximately 1 to 1.5 cm deep. The lumen is lined with sensory neuroepithelia which

constitute a distinct locus of pheromone receptors.

Collaborating otolaryngologists rinsed the human VNO specimens in sterile

phosphate-buffered saline (PBS) immediately after resection to remove blood and other

fluids. They rapidly excised extraneous tissue and snap-froze the VNO in liquid nitrogen.

The frozen specimens were shipped on dry ice to the laboratory for RNA extraction.

5   Thus, authentic VNO tissue specimens were collected under conditions that sought to

minimize potential degradation of the RNA.

Example 2
Isolation of a mRNA from pooled VNO Specimens

10   Total cellular RNA was extracted from the pooled VNO specimens using Trizol

(Life Technologies). This procedure is rapid, and minimizes RNA degradation.

However, any method for RNA isolation may be used.

Tissue samples were homogenized in Trizol Reagent (Life Technologies) using a

glass-Teflon or power homogenizer. After incubation of the homogenized samples for 5

15   minutes at room temperature to permit the complete dissociation of nucleoprotein

complexes, 0.2-ml chloroform was added per 1 ml Trizol Reagent. The samples were

mixed vigorously and then centrifuged at 12,000xg for 15 minutes at 4°C. Centrifugation

separated the biphasic mixtures into the lower red, phenol-chloroform phase and the

upper colorless, aqueous phase.

20   The RNA was precipitated from the aqueous phase by mixing with 0.5 ml of

isopropanol (for each initial milliliter of Trizol Reagent). The samples were incubated at

room temperature for 10 minutes and centrifuged at 12,000xg for 10 minutes at 4°C. The

supernatant was removed and the RNA pellet was washed once with 70% ethanol. The

pellet was air dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water. The

RNA was quantitated by A_{260} measurement.

5

Example 3
cDNA Synthesis for library construction

First-strand cDNA was prepared using SUPERSCRIPT II (RNase H⁻) Reverse

Transcriptase (Life Technologies) which had been optimized for maximum yield of long

cDNA products. The reaction was primed with a Not I-oligo(dT)$_{12-18}$ adapter-primer

10   (Life Technologies) under conditions specified by the supplier. cDNA synthesis was

primed by the oligo(dT)$_{12-18}$ at the 3'-poly(A) end of the mRNA; the adapter adds a Not

I restriction site to the 5'-end of the first-strand cDNA. The reaction was incubated at

45°C to melt potential secondary structures in the template mRNA. The length of first-

strand cDNA that was synthesized in small pilot reaction mixtures containing [α-

15   ^{32}P]dCTP was determined, relative to known DNA standards, by alkaline agarose gel

electrophoresis and autoradiography to test the quality and performance of the materials

and conditions.

Second-strand synthesis was catalyzed by *E. coli* DNA polymerase I in

combination with RNase H and *E. coli* DNA ligase at 16°C. In this procedure, RNase H

20   introduces nicks into the RNA of the mRNA:cDNA hybrids and DNA polymerase I

synthesizes second-strands by nick-translation; the low temperature reduces spurious

synthesis by DNA polymerase I which has a tendency to strand-displace (rather than

nick-translate) at higher temperatures. DNA ligase repairs nicks in the second-strands

and improves the yield of long cDNAs. In the final step, T4 DNA polymerase fills in and

blunts the ends of the double-stranded cDNA. The double-stranded cDNA was then

deproteinized by organic extraction and precipitated with ethanol.

5   An excess of the commercially available Sal I adapter was ligated to the blunt ends

of the double-stranded cDNA from the Not-oligo(dT)-primed reaction. Subsequent

digestion with Not I removed the Sal I adapter from one end yielding molecules with a

Sal I and a Not I end suitable for directional cloning into a vector that has been double-cut

with these two enzymes. The recognition sites for Not I and Sal I are extremely rare in

10   human DNA and thus the double-stranded cDNAs should be cut internally by these

enzymes only very infrequently, if at all.

Unligated adapters, low molecular-weight cDNA (<500 base pairs),

deoxynucleoside triphosphates, etc. were subsequently removed by chromatography on

Sephacryl® S-500 HR prior to ligation into the vector. The >500-bp cDNA was ligated

15   into pCMV-Sport 7.neo (Life Technologies) although any of a number of suitable vectors

could be used. This vector has been developed at Life Technologies for cloning

SUPERSCRIPT cDNA libraries. Among its features are a selectable marker gene for

bacteria (ß-lactamase), T7 and SP6 promoters flanking the multiple cloning site for

synthesis of single-stranded sense and anti-sense cRNAs, a cytomegalovirus (CMV)

20   promoter and SV40 polyadenylation signal for eukaryotic expression of directionally

cloned inserts, and a selectable marker gene for eukaryotic cells (neo^r).

33

After ligation to the vector, the DNA was transformed into a highly competent

strain of *E. coli* such as DH10B (Life Technologies). Recombinants were selected on LB

agar plates for resistance to ampicillin. The library was amplified as described in

Example 4 and plates prepared for colony hybridization.

5

Example 4
Amplification of Primary Library
The primary library was amplified once under semi-solid conditions. Semi-solid

amplification of primary cDNA transformants minimizes representational biases that can

10   occur during the expansion of plasmid cDNA libraries.

Media preparation

2X LB: 20 g Tryptone, 10 g Yeast Extract, 10 g NaCl in 1,000 mls H_2O.

2X LB Glycerol (12.5%): 175 ml 2X LB, 25 ml Glycerol (100%). Filter sterilize

and store for up to two months at room temperature.

15   Prepare 2 liters of 2X LB. Remove 200 mls of the 2X LB to make the 2X LB

Glycerol. Place a large stir bar and 1.35 g SeaPrep (FMC) agarose into each of four 500-

ml autoclavable bottles. Place bottles on stir plates. With the stir plate turned on, add

450 ml of 2X LB to each bottle, avoiding the formation of large clumps of agarose.

Autoclave these bottles of 2X LB agarose for 30 min. Cool bottles in 37°C water bath for

20   approximately 2 hours until media reaches 37°C. After the media reaches 37°C, add

Carbenicillin to 50 µg/ml (preferred antibiotic) or Ampicillin 200 µg/ml. Mix on stir

plate.

Amplification

Briefly, 4×10^5 to 6×10^5 primary cDNA transformants (colonies from original library) were added to each of the autoclaved bottles of 2x LB agarose and mixed thoroughly on a stir plate for 2 minutes. The caps were tightened and the bottles placed in

5   an ice water bath (0°C) such that the level of water in the bath is at the same level as the upper level of media in the bottle. The bottles were incubated for 1 hour in the ice bath. The bottles were gently removed from the ice bath and the excess water wiped off the outside of the bottles. The bottle caps were loosened and the bottles placed in a gravity flow incubator set at 30°C. The bottles were incubated for 40-60 hrs without disturbance.

10   *Cell harvest*

The contents of the bottles were poured into GSA bottles and centrifuged at 8,000 rpm for 20 minutes at room temperature (Caution: Make sure that the rotor was set at room temperature for at least two hours before adding the GSA bottles. Rotors at 4°C will cause solidification of agar.) The supernatant was decanted off and the cells

15   resuspended in a total volume of 100 ml 2X LB Glycerol (12.5%). Two 100 µl aliquots were removed for plating, further analysis, and colony estimate. Cells were filtered through sterile cheesecloth to remove agarose clumps if present.

Cell storage

The cells were subdivided into small aliquots (Note: It is useful to make a number

20   of 1 ml and 100 µl aliquots.) and stored at -70°C. Frozen cells can then be used to

prepare DNA for experiments or can be further amplified in liquid at 30°C to obtain

DNA. Use 2.5×10^9 cells per 100-ml growth medium for further expansion of library.

Amplified library

The amplified library contains $\sim3.5 \times 10^{11}$ colony-forming units (CFU)

5   representing $\sim1 \times 10^7$ primary transformants. Inserts range from ≥300 to >3000 base pairs

(bp) in length, with an average insert size of ~1500 bp. For comparison, mRNAs in the

rat V1R receptor family contain, on average, ~915 bases in the open reading frame (ORF)

and ~230 bases in the 3´-untranslated region (UTR) (Dulac and Axel, 1995). Therefore,

the inventive cDNA library will be a source of suitably sized clones for identification and

10   characterization of numerous genes and gene fragments. We also point out that full-

length cDNAs containing the precise 5´ end of the mRNA sequence, though scientifically

interesting, are not essential provided that we obtain the entire full-length ORF (see

below).

15   Example 5
                          Isolation of RNA from individual VNO specimens
          RNA was isolated from individual VNO specimens using the Qiagen (Carlsbad,

CA) RNeasy kit according to the manufacturer's instructions. In brief, each VNO

specimen, frozen in liquid nitrogen, was ground with a disposable pestle in Lysis Buffer

20   provided in the kit and then further processed according to the manufacturer's

instructions. The RNA was treated with DNase and then eluted from the matrix with

Elution Buffer provided in the kit . It was stored frozen at –80°C.

Example 6
RT-PCR

First-strand cDNA was prepared from RNA isolated from individual VNOs using the

Roche Molecular (Indianapolis, IN) First-Strand cDNA Synthesis kit. In brief, an aliquot

5 of VNO RNA was mixed with the appropriate buffer, salts, Mg^{2+}, deoxynucleotide

triphosphates, RNase inhibitor, random hexamer primers, and AMV reverse transcriptase

(RT) as provided in the kit. The reaction mixture was incubated at 25°C for 10 min to

anneal the primers and then at 42°C for 60 min to synthesize the cDNA. The mixture

was heated at 99°C for 5 min to inactivate the RT. The resulting cDNA was diluted with

10 sterile H_2O and stored at -20°C. As a control, a reaction mixture without added RT was

processed in parallel.

PCR primer pairs were designed based on the GenBank entry for the target

sequence using readily available software such as Primers. Biosource (Foster City, CA)

synthesized the primers on a standard fee-for-service basis. The primers were within or

15 flanking the coding region of the target sequence. The PCR reaction contained the

appropriate buffer, salts, Mg^{2+}, deoxynucleotide triphosphates, primers, and either

Expand High Fidelity polymerase (Roche Molecular) or Pfu polymerase (Stratagene; La

Jolla, CA). Amplification was performed in a Perkin Elmer 9600 thermocycler. For

internal primers and Expand polymerase, the conditions were: 94°C for 2 min followed

20 by 40 cycles of 94°C for 15 sec, 56°C for 30 sec, 72°C for 45 sec, and one cycle of 72°C

for 5 min and 4°C for 5 min. The template was female or male first-strand VNO cDNA

or plasmid DNA from isolated from the female VNO cDNA library; aliquots of the

minus-RT reactions were amplified in parallel as a negative control for the first-strand

cDNA.

For flanking primers and Pfu polymerase, the conditions were: 94°C for 2 min

followed by 40 cycles of 94°C for 15 sec, 54°C for 30 sec, 72°C for 1 min, and one cycle

5   of 72°C for 5 min and 4°C for 5 min. The template was female or male VNO first-strand

cDNA.

An aliquot of each reaction was analyzed on an agarose gel containing ethidium

bromide. The amplicons were isolated from the gel, ligated into pGEM-T-Easy

(Promega; Madison, WI), and transformed into competent E. coli DH10B (Life

10   Technologies; Rockville, MD). Transformants were selected on agar plates containing

ampicillin. Colonies were screened for the presence of a plasmid with an insert of the

expected size. Selected plasmids were sequenced to identify the amplicon and the

encoded open reading frame.

Example 7
Sequencing
15
Sequencing of selected (full-length) clones was done by standard methods.

Oligonucleotides that are complementary to the T7 and SP6 promoters in the vector were

used to prime sequencing reactions from the ends of a cloned insert. Internal primers,

based on newly acquired sequence data, were synthesized, as necessary, to sequence

20   overlapping internal regions of the cloned cDNAs.

We examined the assembled sequences by computer for the presence of a potential

full-length open reading frame. We used standard BLAST analysis to compare the

human VNO clone to sequences in GenBank. The human hVNO-R1 (SEQ ID No. 1)

clone shows homology to genes encoding the superfamily of G protein-coupled receptors

5   and it predicts 7 transmembrane domains.

The nucleotide sequence of the hVNO-R1 cDNA (SEQ ID No. 1) is 1114

nucleotides in length containing two in-frame initiation sites at residues 17 and 44 and a

termination codon at 1088. The two initiation sites yield two different forms of the

receptor - a long (SEQ ID No. 3) and short (SEQ ID No. 4) form of the receptor. The

10   initiation site located at residue 17 results in the long form of the receptor. The long form

is a 357 amino acid residue protein having the amino acid sequence shown in Figure 2

(SEQ ID No. 3). The short form has its initiation codon at residue 44 of Figure 1. The

short form is a 348 amino acid residue protein having the amino acid sequence shown in

Figure 3 (SEQ ID No. 4). The nucleic acid sequence of the short form is given in SEQ ID

15   No. 7 or 8 (both encode the same protein).

The long form of the hVNO-R1 receptor was analyzed by computer for

transmembrane motifs. Similar to other G protein-coupled receptors this form was found

to have 7 regions that are each likely to correspond to a transmembrane region. Figure 4

shows the amino acid sequence of the long form of the hVNO-R1 receptor with the seven

20   theoretical transmembrane domains underlined and indicated as TM1 – TM7.

A second, mutant allele of the hVNO-R1 receptor gene was identified, mhVNO-R1 (SEQ ID No. 5). The cDNA clone begins with an initiation codon that corresponds with the first initiation codon of the hVNO-R1 clone (SEQ ID No. 1) at residue 17.

The sequence of mhVNO-R1 (SEQ ID No. 5), when aligned with hVNO-R1, is

5   identical to hVNO-R1 except for two base differences. At residue 136 of SEQ ID No. 5 there is a C→T mutation that results in a nonsense or stop codon. Thus, translation of the mhVNO-R1 gene would result in a truncated VNO receptor protein (SEQ ID No. 6) that would be of limited functionality, if any. At position 762 of SEQ ID No. 5, mhVNO-R1 has an A residue whereas hVNO-R1 (SEQ ID No. 1) has a G at the corresponding

10   position, i.e., position 778 of SEQ ID No. 1.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended

15   claims.